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Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a member of the EGF family which binds to and activates the EGF receptor. Its role in breast cancer, however, is unclear. We have studied the mechanism of induction of HB-EGF by EGF in the spontaneously immortalized mammary epithelial cell line MCF-10A and in MCF-10A cells that have been transformed with oncogenic Ha-ras. The levels of EGFR, erbB-2 and erbB-3 were very similar in the nontransformed and in the transformed MCF-10A cells. However, the levels of mitogen-activated protein kinase (MAPK) were elevated in the MCF-10A cells transformed with the Ha-ras oncogene. Neutralizing antibodies and inhibitors against the EGFR were able to inhibit the induction of HB-EGF mRNA levels in the nontransformed MCF-10A cells but not in the MCF-10A cells transfected with oncogenic Ha-ras. However, an inhibitor of MAPK kinase (MEK) completely blocked the EGF induction of HB-EGF mRNA levels in the parental MCF-10A cells as well as in transformed MCF-10A cells, which suggests that EGF may be operating through other erbB receptors such as erbB-2/erbB-3 heterodimers and that MAPK is involved in the signaling pathway of HB-EGF induction in nontransformed and transformed human mammary epithelial cells.

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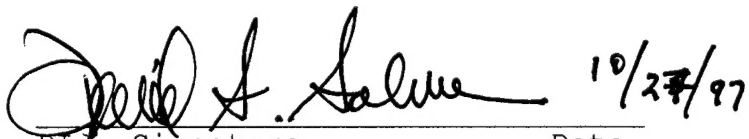

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## TABLE OF CONTENTS

## INTRODUCTION

Heparin-binding epidermal growth factor (HB-EGF) is a member of the EGF family which binds to the EGF receptor. It was originally isolated from conditioned media of the human monocyte-like U-937 cells that were induced with 12-O-tetradecanoylphorbol-13-acetate (TPA) (Higashiyama *et al.*, 1991). Little is known about the role of HB-EGF in breast cancer, which is the scope of this project. Others members of the EGF family such as transforming growth factor alpha (TGF-α) and amphiregulin (AR) are known to be induced by TPA in breast cancer cells and also by steroid hormones such as 17-β-estradiol (E2). We analyzed whether HB-EGF was induced by these agents in nontransformed and transformed human mammary epithelial cells. TPA was able to induce HB-EGF mRNA levels in the cell lines analyzed. However, estrogen and progesterone did not induce HB-EGF mRNA in the cell lines analyzed.

It has been shown that EGF-related peptides can also be induced by EGF (Barnard *et al.*, 1994). We have studied the auto and cross-induction of HB-EGF with all the EGF-related peptides to determine whether there is any differential mechanism of induction between these peptides in nontransformed human mammary epithelial cells. All the EGF related peptides except heregulin beta-1 (HRG-β1) were able to induce HB-EGF mRNA levels, EGF being the most potent activator. We have studied the signaling pathway by which EGF induces HB-EGF expression in nontransformed and transformed human mammary epithelial cells. We want to determine whether EGF is acting solely through the EGFR or some of the other erbB receptors by heterodimerization.

It has been recently shown that HB-EGF is an early responsive gene that can be activated by the ras/raf signaling pathway (McCarthy *et al.*, 1995). Furthermore, phosphorylation of Ets-2 by oncogenic raf-1 accompanies activation of p42 (ERK2) and p44 (ERK1) mitogen-activated protein kinase (MAPK) and induction of HB-EGF transcription (McCarthy *et al.*, 1997), suggesting that MAPK is implicated in the regulation of HB-EGF. We wanted to test this hypothesis in human transformed mammary epithelial cells that have been transfected with the ras oncogene (Basolo *et al.*, 1991). We wanted to determine whether MAPK is involved in the pathway of induction of HB-EGF transcription by EGF in nontransformed and transformed human mammary epithelial cells.

## EXPERIMENTAL METHODS

Reagents: Human mammary epithelial cells were obtained from Dr. Samuel Brooks, Michigan Cancer Foundation, Detroit, MI, the American Type Culture Collection (ATCC), Rockville, MD, and from Dr. Marc Lippman, Lombardi Cancer Center, Georgetown University, Washington, D.C. The BS-HBE clone was constructed by subcloning a 402-bp restriction fragment of the HB-EGF cDNA into the Eco RI-Kpn I site of the pBC KS- polylinker region. The pGEM-AR and p36B4 clones have already been described (Martínez-Lacaci *et al.*, 1995; Saceda *et al.*, 1988). The pUC (7-1) clone containing a 1.1 Kb HB-EGF cDNA fragment was obtained from Dr. Judith Abraham, Scios Nova Inc., Mountain View, CA. An anti-goat neutralizing antibody against HB-EGF was purchased from R&D Systems, Minneapolis, MN. The anti-phosphotyrosine monoclonal antibody PY20 was purchased from Transduction Laboratories, Lexington, KY. The anti-phosphotyrosine monoclonal antibody 4G10 was purchased from Upstate Biotechnology Inc., Lake Placid, NY. Rabbit and goat polyclonal antibodies against HB-EGF, EGFR, c-neu, erbB-3 and erbB-4 were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA. The phospho-specific antibodies against MAPK and Elk-1, the antibodies against p44/42 MAPK and Elk-1 and the Elk-1 fusion protein were purchased from New England Biolabs, Inc., Beverly, MA. The monoclonal antibody LA069 against Ha-ras was purchased from Quality Biotech, Camden, NJ. The anti-EGF receptor monoclonal antibody 225 (Gill *et al.*, 1984) was obtained from Dr. John Mendelsohn, MD Anderson Cancer Center, University of Texas, Houston, TX. The DAPH-1 compound was obtained from Ciba-Geigy Limited, Basle, Switzerland. The PD-98059 compound was purchased from Calbiochem, La Jolla, CA.

Cell culture: Nontransformed and transformed human mammary epithelial cells were grown in DMEM/HAMF12 supplemented with 5% horse serum, 10 U/ml penicillin-10 g/ml streptomycin, 0.5 g/ml hydrocortisone, 5 g/ml insulin, 0.1 g/ml cholera toxin and 20 ng/ml EGF. When cells were 50-60% confluent, medium were replaced to complete medium except for EGF and maintained for 3-4 days. Subsequently, medium was replaced to basic medium without EGF, horse serum and insulin for 48 hr and cells were treated with different peptides, antibodies or drugs for appropriate times.

Isolation of RNA: Total cellular RNA was isolated from nontransformed and transformed human mammary epithelial cells using the Perfect RNA total RNA isolation kit (5 Prime-3 Prime, Inc, Boulder, CO) and stored at -70°C. RNA was dissolved in 70% ethanol and the

optical density at 260 and 280 nm was determined.

RNase protection assay: 32P-labeled antisense riboprobes were in vitro synthesized from BS-HBE, pGEM-AR and p36B4 using T3, SP6 and T7 polymerases, respectively. Subsequently, 60-µg aliquots of total RNA isolated from nontransformed and transformed human breast epithelial cells were hybridized for 12-16 hr at 50°C, and treated with RNase A for 30 min at 25°C. The protected fragments were electrophoresed on a 6% polyacrylamide gel, which was subsequently dried and exposed to autoradiography.

Western blot analysis: Nontransformed and transformed human mammary epithelial cells were lysed using lysis buffer containing 20 mM Tris pH 7.5, 100 mM NaCl, 1% Nonidet-P40, 0.5% deoxycholate, 5 mM $MgCl_2$ and proteinase inhibitors: leupeptin (5 µg/ml), aprotinin (20 µg/ml) and phenylmethylsulfonyl fluoride (35.8 µg/ml) and incubated for 15 min at 4°C. Alternatively, conditioned media were collected and concentrated using Centripep concentrators. Protein concentration of the samples was determined and 80 µg were subjected to Tris-Gly SDS-PAGE, transferred to PVDF membranes, blocked with Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST) and 3% bovine serum albumin (BSA) for 1-3 hr, incubated with appropiate antibodies for 3-16 hr , washed, incubated for 1 hr with a secondary antibody linked to horseradish peroxidase, washed, incubated with enhanced chemiluminescence (ECL) reagents for 1 min and exposed to autoradiography.

c-erbB receptor phosphorylation assay: Nontransformed and transformed human mammary epithelial cells were grown in 100-mm dishes. When they were 50% confluent, medium was replaced with complete medium without EGF for 3-4 days and subsequently, cells were serum-starved for 48 hr. Cells were treated with EGF, antibodies or drugs for 10 min, lysed with lysis buffer containing 1% Triton X-100, 10 mM Tris, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride and 1 mM sodium orthovanadate and proteinase inhibitors for 15 min at 4°C. Cells were scraped, centrifuged at 14,000 x g for 15 min at 4°C and supernatant was transferred to a clean tube. Subsequently, protein concentration of the samples was determined and 300 µg of protein

were incubated with 1 μg of antibodies against EGF receptor, c-erbB-2, c-erbB-3 or c-erbB-4 and rotated end over end for 3-16 hr at 4°C. Subsequently, samples were incubated with protein G Sepaharose beads and rotated for 1 hr at 4°C. Beads were washed three times with lysis buffer, dried, resuspended in lysis buffer. Proteins were eluted by boiling the beads for 7 min and by centrifugation at 14,000 x g for 15 min. Samples were subjected to 6 % SDS-PAGE, transferred to PVDF membranes, blocked with 3% BSA-TBST and incubated with anti-phosphotyrosine antibodies (PY20 and 4G10) or antibodies against erbB receptors overnight. Membranes were washed, incubated with a secondary antibody linked to horseradish-peroxidase, washed, incubated with ECL reagents for 1 min and exposed to autoradiography.

MAP kinase assay: MCF-10A nontransformed and transformed cells were lysed as described above for Western blot analysis, protein concentration was determined and 80 μg were subjected to 4-20% SDS-PAGE. Subsequently, samples were transferred to PVDF membranes, blocked with 3% BSA-TBST, incubated with a phospho-specific anti-MAPK antibody for 3-16 hr at 4°C, washed, incubated with a secondary antibody coupled to horseradish-peroxidase for 1 hr, washed and incubated with ECL reagents for 1 min and exposed to autoradiography. Blots were stripped, blocked with 3%BSA-TBST and incubated with an anti-p44/42 MAPK activity to determine levels MAPK protein.

MAP kinase activity assay: Nontransformed and transformed human mammary epithelial cells were lysed in phosphorylation buffer. Subsequently, 300 μg of protein were incubated with a phospho-specific anti-MAPK antibody and rotated overnight at 4°C. Active MAPK was used as a positive control in these experiments. Subsequently, protein G sepahrose beads were added and the mixture was rotated for 1-3 hr. Beads were washed and resuspended in a kinase buffer containing 25 mM Tris pH 7.5, 5 mM -glycerolphosphate, 2 mM DTT, 0.1 mM sodium orthovanadate and 10 mM $MgCl_2$. Subsequently, 100 μM ATP and 1 μg Elk-1 fusion protein were added and samples were incubated at 30°C for 30 min. Sample buffer was added, samples were boiled and subjected to 4-20% SDS-PAGE. Samples were transferred to PVDF membranes, blocked with 3% BSA-TBST and incubated with a phospho-specific anti-Elk-1 antibody overnight at 4°C. Filters were washed, incubated with a secondary antibody linked to horseradish-

peroxidase for 1 hr, washed, incubated with ECL reagents and exposed to autoradiography. To normalize Elk-1 protein levels, filters were stripped, blocked with 3%-BSA-TBST and incubated with an anti-Elk-1 antibody.

Anchorage-independent growth assay: Nontransformed and transformed human mammary epithelial cells were suspended in 0.36% agar supplemented with the appropiate medium and seeded over a 0.8% agar base layer. After 14 days, cells were stained with nitroblue tetrazolium and colonies larger than 50 mm were counted on an Artek Colony Counter.

## RESULTS

Effects of EGF-related peptides on HB-EGF mRNA levels: The spontaneously immortalized, nontransformed human mammary epithelial cell line, MCF-10A, was treated with different EGF-related peptides to analyze their effect on the induction of HB-EGF mRNA levels for 1, 3, 6 and 24 hr. Total RNA was isolated and analyzed by RNase protection. HB-EGF mRNA was induced with the following peptides (10 ng/ml): EGF, TGF-α, AR, HB-EGF, and betacellulin (BTC). TPA was used as a positive control. HB-EGF was induced very rapidly with the EGF-related peptides. However, the induction of HB-EGF with EGF seemed to be more sustained compared to the effect of the other peptides (Fig.1).

Induction of HB-EGF protein and its secretion in MCF-10A cells: MCF-10A cells were induced with EGF for 3, 6, 24, 48 and 72 hr. Levels of HB-EGF protein were detected in MCF-10A lysates (LYS) as well as in their conditioned media (CM) by immunoprecipitation with an anti-HB-EGF polyclonal antibody followed by Western blot analysis with a different anti-HB-EGF antibody. HB-EGF protein was induced within 3 hr and its secretion within 6 hr (Fig. 2).

Levels of HB-EGF in transformed MCF-10A cells: MCF-10A cells were transformed with c-Ha-ras (N-ras) and a point mutated form of Ha-ras (Ha-ras) from which subclones were derived (T2). Levels of HB-EGF mRNA were measured by RNase protection in different clones and compared to the parental cell line MCF-10A. Most of the clones showed enhanced levels of HB-EGF mRNA (Fig.3).

Levels of Ha-ras protein in MCF-10A transformed cells: Levels of p21 (Ha-ras) protein were analyed by Western blot in nontransformed MCF-10A cells and in MCF-10A cells transfected with the Ha-ras protooncogene or with a point-mutated Ha-ras form. The transfected cells showed higher levels of p21 (Ha-ras) protein compared to the parental MCF-10 cells (Fig. 4).

Anchorage-independent growth of MCF-10A transformed cells: The colony formation ability of the MCF-10A cells transfected with Ha-ras was measured and compared to the parental MCF-10A cells. Only the cells transfected with the point-mutated Ha-ras but not the normal Ha-ras protooncogene were able to grow in soft agar and form colonies. (Fig.

5).

Levels of EGFR, erbB-2 and erbB-3 in MCF-10A transformed cells: Levels of EGFR, erbB-2 and erbB-3 were measured by Western blot analysis in MCF-10A cells and in MCF-10A transformed with the normal Ha-ras protooncogene (N-ras) and with a point mutated Ha-ras oncogene. All the clones had similar levels of receptors except for the T2E clone. Additionally, the N-ras cells had less EGFR protein compared to the normal nontransformed MCF-10A cells (Fig. 6).

Levels of MAP kinase and its activity in MCF-10A transformed cells: Levels of p44/42 MAPK (p42) and their phosphorylated forms (pp42/44) were measured by Western blot in MCF-10A cells transfected with Ha-*ras* (N-ras) or an activated form of Ha-*ras* (Ha-ras) from which subclones were derived (T2) and compared to the parental cell line MCF-10A. Some of the clones transfected with the Ha-*ras* oncogene showed higher levels of p42 (ERK2) protein (Fig. 7). Additionally, MAPK activity was measured in the same cells using Elk-1 as a substrate. Elk-1 belongs to the Ets family of transcription factors and it is a direct target of MAPK. Levels of pElk-1 and Elk-1 were determined by Western blot anaysis and bands were quantified by scanning densitometry. The transformed MCF-10A expressing the Ha-ras oncogene showed higher levels of MAPK activity compared to the parental MCF-10A cells (Fig. 8).

Blocking of the EGF receptor: MCF-10A cells and cells transfected with the normal Ha-*ras* protooncogene (N-ras) and subclones derived from cells transfected with an activated Ha-*ras* oncogene (T2) were preincubated with the 225 neutralizing antibody against the EGF receptor (10 μg/ml) and treated with EGF (10 ng/ml) for 1 hr. HB-EGF mRNA levels were analyzed by RNase protection. The inducible effect of EGF was blocked in the MCF-10A parental cells. However, in the MCF-10A cells transfected with the Ha-*ras* oncogene the effect of EGF was not blocked and in some of the clones was increased (Fig. 9). Likewise, MCF-10A, N-ras and T2B cells were pretreated with the EGFR tyrosine kinase inhibitor DAPH-1 (50 μM) for 1 hr, prior to 1 hr treatment with EGF and similar results were obtained (Fig. 10). In order to determine whether the 225 monoclonal antibody was indeed blocking the activation of the EGFR, MCF-10A cells and the T2B ras clone were pretreated with the 225 Ab (10 μg/ml) for 30 min and cells were challenged with EGF (100 ng/ml) for 10 min. Phosphorylation of the EGF was determined by immunoprecipitating the EGFR followed by Western blot analysis with anti-P-Tyr antibodies (Fig. 13). The 225

monoclonal antibody was able to block phosphorylation of EGFR.

Blocking of MAPK: MCF-10A cells and cells transfected with the normal Ha-*ras* protooncogene (N-ras) and a subclone derived from cells transfected with an activated Ha-*ras* oncogene (T2B) were preincubated with the MEK inhibitor PD-98059 (30 μM) for 1 hr and treated with EGF (10 ng/ml) for 1 hr. HB-EGF mRNA levels were measured by RNase protection. PD-98059 completely abolished the EGF induction of HB-EGF mRNA levels in all the cell lines analyzed (Fig. 10). In order to determine whether this MEK inhibitor could inactivate p44/42 MAPK, MCF-10A cells were pretreated with PD-98059 (30 μM) for 30 min prior to treatment with EGF (100 ng/ml) for 10 min. Levels of pp44/42 and p44/42 were determined by Western blot (Fig. 11). Phosphoryation of MAPK was inhibited by the MEK inhibitor PD-98059.

Phosphorylation of erbB receptors: Levels of phosphorylation of EGFR and erbB-2 were measured in MCF-10A cells and in MCF-10A cells transfectd with Ha-ras or with a point mutated from of Ha-ras oncogene after treating the cells with EGF (100 ng/ml) for 10 min. EGFR was immunoprecipitated with an anti-EGFR antibody and subjeceted to Western blot analysis with anti-P-Tyr antibodies (Fig. 12). The N-ras cells which have less EGFR protein showed lower levels of EGFR phosphorylation, followed by the T2E clone. The rest of the clones transfected with oncogenic ras showed higher leves of phosphorylated EGFR. Likewise, erbB-2 was immunoprecipitated with an anti-erbB2 antibody after treating the cells with EGF (100 ng/ml) for 10 min. Phosphorylation of erbB-2 was detected by Western blot with anti-P-Tyr antibodies and levels of erbB-2 protein with an anti-erbB-2 antibody (Fig. 14). The phosphorylation of erbB-2 levels were similar in all the cells examined.

## DISCUSSION

In this project the role of HB-EGF in breast cancer is being studied. In relation to the Statement of Work outlined in the proposal the following tasks have been attempted:

Task 1: *To analyze the HB-EGF mRNA and proteins levels in normal, benign and malignant breast tissues and in nontransformed and malignant human mammary epithelial cells.* The basal levels of HB-EGF mRNA have been analyzed in several transformed and nontransformed human mammary epithelial cells. These data was included in the annual report of October 1996. In this report we have shown that HB-EGF mRNA levels are higher in transformed human mammary epithelial cells that have been transfected with oncogenic Ha-ras than in nontransformed mammary epithelial cells (Fig. 3). We solved the problem we had last year in order to detect HB-EGF protein by using a different approach. We immunoprecipitated 300 μg of protein with a polyclonal antibody raised against HB-EGF (Santa Cruz Biotechnolgy) and performed a Western blot with a different polyclonal antibody against HB-EGF (R&D Systems). We have been able to detect HB-EGF protein in the lysates and conditioned media of the nontransformed mammary epithelial MCF-10A cells (Fig. 2) as well as in the breast cancer MDA-MB-231 cells (data not shown). Some breast tumor specimens and their adjacent non-involved tissue have been analyzed for HB-EGF and BTC expression by RT-PCR (data not shown). We want to analyzed more specimens before drawing any conclusions.

Task 2: *To measure the HB-EGF mRNA and protein levels in nontransformed and malignant human mammary epithelial cells after treatment with steroid hormones, differentiating agents and growth modulators.* HB-EGF was not induced by either estrogen or progesterone in any of the estrogen responsive, ER positive breast cancer cells analyzed. However, TPA was able to induce HB-EGF mRNA levels in all the cell lines analyzed with the exception of MDA-MB-453 cells. These data was presented in the annual report of October 1996. Induction of HB-EGF was analyzed in MCF-10A cells with different EGF-related peptides and with TPA (Fig. 1). EGF is the most potent activator after long periods of time. Additionally HB-EGF protein levels and its secretion are induced in the nontransformed mammary epithelial MCF-10A cells after EGF treatment (Fig. 2). We are studying the mechanism of induction with EGF and TPA and determining whether there is a cross-talk between these two factors. We are in the process of concluding these experiments by using inhibitors of the EGF receptor (neutralizing antibodies, tyrphostins, etc) and protein kinase C inhibitors (bisindolylmaleimide I, bryostatin and H-7). The

neutralizing monoclonal 225 antibody against the EGFR (Gill *et al.*, 1984) is able to block the EGF induction of HB-EGF mRNA levels in MCF-10A cells (Fig. 9). Additionally, the 225 antibody is able to block the TPA induction of HB-EGF mRNA levels in MCF-10A cells (data not shown), suggesting that EGF and TPA are operating through the same signaling pathway. Some preliminary data using the PKC inhibitor bisindolylmaleimide I suggests that EGF and TPA may be using the same pathway. However, these data must be corroborated using other PKC inhibitors.

We have studied the signaling mechanism by which EGF upregulates HB-EGF expression in nontransformed and transformed mammary epithelial cells that have been transfected with the Ha-ras oncogene. We think that using this system is very relevant since it has been reported that HB-EGF expression is activated by the ras/raf signaling pathway (McCarthy *et al.*, 1995). We have characterized the nontransformed human mammary epithelial MCF-10A cells and the MCF-10A cells that have been transfected with the Ha-ras protooncogene (N-ras) and with a point mutated form of Ha-ras (Ha-ras) (Basolo *et al.*, 1991). The T2 series were subclones derived from the Ha-ras cells by selecting colonies that were able to grow in soft agar. The levels of p21 ras protein are higher in the cells transfected with Ha-ras (Fig. 4) compared to the parental MCF-10A cells. The T2 and the T2D clone show higher levels of p21 ras protein. The levels of erbB receptors were also analyzed in these cells (Fig. 6). With the exception of the N-ras cells that have less EGFR and the T2E clone that has lower levels of EGFR, erbB-2 and erbB-3 (it may be due to a loading error), the rest of the clones seem to have similar levels of erbB receptors compared to the parental MCF-10A cells. These results indicate that transformation with ras does not induce expression of erbB receptors. The fact that the N-ras cells have less EGFR is not understood. Overexpression of Ha-ras in these cells seems to downregulate expression of EGFR. It would be interesting to determine whether K-ras or N-ras have the same effect as Ha-ras in MCF-10A cells. Further work is required in order to demonstrate this finding. The levels of erbB-4 in the normal MCF10A cells and in the transfectants are not significant. We have performed Western blot using different antibodies and the levels of erbB-4 are almost undetectable in all the cells analyzed (data not shown).

It is known that activation of ras leads to activation of ERK 1/2 MAPK, which has been implicated in oncogenesis (Hunter, 1997). Additionally, it has been shown that MAPK is overexpressed and is heavily phosphorylated in breast carcinomas compared to normal breast tissue, benign fibroadenomas or fibrocystic disease (Sivaram *et al.*, 1997). We have analyzed the levels of MAPK and its activity in nontransformed and transformed MCF-10A cells. We suspect that MAPK activity is upregulated in MCF-10A cells transformed with the oncogenic ras. The levels of p42 (ERK2) MAPK are higher in cells

transfected with oncogenic ras than in the parental MCF-10A cells or the N-ras cells (Fig. 7). However the levels of phosphorylated p42 (pp42) are almost the same. The levels of p44 (ERK1) in these cells are very low and difficult to detect in the absence of EGF or serum (data not shown). However, the levels of MAPK activity are higher in the cells transfected with ras compared to the parental MCF-10A cells (Fig. 8). These experiements are difficult to perform because the basal levels of MAPK fluctuate between experiments due to cell confluency, presence of serum or some other factors. We have performed this assay several times and in general, the activity of MAPK is higher in the ras transfected MCF-10A cells. It is known that MAPK translocates to the nucleus once it is activated, where it can phosphorylate and activate a variety of transcription factors such as Elk-1 (Hunter, 1997). We are planning to detect subcellular localization of p44/p42 MAPK by immunocytochemistry in order to determine whether in the ras-transformed MCF-10A cells p44/42 MAPK is already localized in the nucleus in the abscence of external stimuli such as EGF.

In order to determine whether EGFR is implicated in the induction of HB-EGF by EGF, nontransformed MCF-10A cells and MCF-10A cells transformed with Ha-ras were preincubated with the anti-EGFR monoclonal 225 antibody prior to EGF treatment and RNA levels were analyzed by RNase protection (Fig.9). Likewise, cells were pretreated with DAPH-1, a protein tyrosine kinase inhibitor specific for EGFR (Buchdunger *et al.*, 1994), prior to EGF treatment and RNA was analyzed by RNase protection (Fig. 10). The induction of HB-EGF was blocked in the parental MCF-10A cells as well as in the N-ras. However, EGF was able to stimulate HB-EGF expression by an EGFR independent mechanism in the MCF-10A cells transformed with oncogenic Ha-ras. The effect of the 225 antibody on phosphorylation of EGFR in MCF-10A cells and the T2B clone were determined (Fig. 13). In both cases, the 225 antibody was able to block phosphorylation of EGFR. Further experiments have been carried out using tyrphostins specific for the EGFR tyrosine kinase, such as AG-1478 and B-42 (Levitzki and Gazit, 1995) and similar results were found (data not shown).

In order to determine whether ERK1/2 MAPK is involved in the induction of HB-EGF by EGF, nontransformed MCF-10A cells and MCF-10A cells transformed with Ha-ras were preincubated with the MEK inhibitor PD-98059, prior to EGF treatment and RNA was analyzed by RNase protection (Fig. 10). PD-98059 was able to block the HB-EGF induction by EGF in MCF-10A cells, N-ras and the T2B clone, suggesting that MAPK is involved in the pathway. In order to demonstrate that PD-98059 inhibits p44/42 MAPK, MCF-10A cells were incubated with EGF in the presence of absence of PD-98059 (Fig. 11). The MEK inhibitor was able to block phosphorylation of p44/p42 MAPK.

These findings indicate that even though EGF has a preference to bind to EGFR, it may be able to interact with other erbB receptors when EGFR is blocked. Different reports have shown that heterodimer formation between EGFR and any of the other erbB-related receptors occurs (Pinkas-Kramarski *et al.*, 1996) and that erbB-2 seems to be the preferred heterodimerization partner (Graus-Porta *et al.*, 1997). Furthermore, secondary dimerization between different erbB receptors can take place upon ligand stimulation (Gamett *et al.*, 1997). EGF treatment can yield erbB-2/erbB3 secondary dimers. Additionally, it has been shown that EGF can mediate signal transduction in cells co-expressing erbB-2 and erbB-3 (Alimandi *et al.*, 1997). We want to determine whether erbB-2 or erbB-3 are involved in the signaling pathway by which EGF stimulates HB-EGF expression. We are planning to use neutralizing antibodies and specific tyrphostins for erbB-2 and erbB-3.

Task 3: *To characterize the mechanism of regulation of HB-EGF (transcriptional or post-transcriptional) after these treatments.* It has been shown that Ets-2 may be activating transcription of HB-EGF by oncogenic raf-1 and that MAPK activity seems to be associated with this phenomena (McCarthy et al., 1997) However, it needs to be demonstrated whether MAPK directly phosphorylates Ets-2, which in turn activates the HB-EGF promoter in the intact cells. It would be interesting to know whether Ets-2 activates HB-EGF transcription in the transformed MCF-10A cells that have been transfected with the Ha-ras oncogene.

Task 4: *To study the effect of HB-EGF on the ADG and AIG of nontransformed or transformed human mammary epithelial cells with activated oncogenes and of established human breast cancer cell lines to delineate how these effects might be modulated by such oncogenes.* Some preliminary studies showed that HB-EGF (10 ng/ml) was able to induce colony formation of the estrogen responsive, ER positive cell lines ZR-75 and T-47D at a greater extent than EGF or AR and it was as potent as E2. Similar results have already been reported in T-47D cells (Beerli and Hynes, 1996). MCF-10A cells transfected with a point mutated form of Ha-ras are able to form colonies in soft agar compared to the parental MCF-10A cells and MCF-10A transfected with the Ha-ras protooncogene (Fig. 5). We are in the process of analyzing the T2, T2A and T2E clones. Since we have determined that HB-EGF expression is higher in the cells transfected with the Ha-ras oncogene and that the ras/raf/MAPK pathway seems to be implicated in this process, we want to assess whether blocking ras with farnesyl inhibitors or MAPK with the PD-98059 compound are able to inhibit colony formation of the transformed mammary epithelial cells.

Task 5: *To determine whether HB-EGF can bind to and phosphorylate other receptors related to the EGF receptor (c-erbB2, c-erbB3, c-erbB4) in nontransformed and malignant human mammary epithelial cells.* We have measured the autophosphorylation of

these receptors upon stimulation of HB-EGF and other EGF-related peptides in MCF-10A, T-47D and MDA-MB-453. HB-EGF and BTC were able to stimulate phosphorylation of erbB-3, as it has been reported (Beerli and Hynes, 1996). We are planning to continue these experiments using different conditions and cell lines in order to demonstrate whether HB-EGF is using a different signaling pathway than the other EGF-related peptides in this system. Some of this data was presented in the annual report of October 1996.

Additionally, we have determined whether EGF can phosphorylate EGFR and erbB-2 in nontransformed MCF-10A cells and in MCF-10A cells transfected with Ha-ras. The phosphorylation levels of EGFR after EGF treatment were determined by immunoprecipitation with an anti-EGFR antibody followed Western blot with anti-P-Tyr antibodies (Fig. 12). N-ras cells, which have low levels of EGFR had low levels of phosphorylated EGFR, followed by the T2E clone. The rest of the ras-transfected clones had higher levels of phosphorylated EGFR than the parental MCF-10A cells. However, the levels of phosphorylated erbB-2 after EGF treatment were almost the same in all the cells (Fig. 14). Interestingly, two bands can be seen in the Western blot performed with anti-P-Tyr antibodies, suggesting that erbB-2 may be forming a heterodimer with a diferent erbB receptor (probably erbB-3) upon EGF stimulation. Further experiments need to be carried out in order to determine phosphorylation of erbB-3 after EGF treatment. Some experiments have been performed in order to detect heterodimer formation or secondary dimerization events among erbB receptors after EGF treatment. However, some difficulties have been encountered since EGFR is not very stable and is rapidly internalized upon EGF treatment.

## CONCLUSIONS

In this project we have studied the regulation of HB-EGF expression in nontransformed and transformed mammary epithelial cells. In the report of October 1996 we included data showing that HB-EGF was induced by TPA in all the cells lines analyzed except in the MDA-MB-453 cells. In contrast with TGF-α or AR, HB-EGF mRNA levels were not induced by estrogen or progesterone. Additionally, HB-EGF mRNA levels were induced in the nontransformed MCF-10A cells with EGF-related peptides. The induction with EGF, however, seemed to be more sustained compared to the other peptides. HRG-β1 or stromal-derived growth factors such as keratinocyte growth factor or hepatocyte growth factor did not have any effect. AR mRNA levels were also induced with EGF-related peptides in a similar fashion.

In this report we have determined the signaling pathway that takes place upon EGF stimulation of HB-EGF expression. HB-EGF has been shown to be induced by the ras/raf signaling pathway. We have analyzed HB-EGF mRNA levels in MCF-10A cells transfected with a normal Ha-ras protooncogene (N-ras) and a point mutated Ha-ras oncogene. In most of the ras clones, HB-EGF mRNA levels were induced compared to the parental MCF-10A cells. We have determined the levels of erbB receptors in these cells. The levels of EGFR, erbB-2 and erbB-3 seem to be very similar in all the cells, with the exception of N-ras, in which EGFR appears to be downregulated. The levels of ERK 1/2 MAPK and its activity are elevated in the MCF-10A cells transformed with the Ha-ras oncogene, compared to the parental MCF-10A cells. The EGF induction of HB-EGF mRNA levels was blocked with EGFR inhibitors (225 antibody, DAPH-1 and tyrphostins) in the MCF-10A and N-ras cells. However, EGF seems to induce HB-EGF mRNA levels by an EGFR-independent mechanism in the MCF-10A cells transformed with oncogenic Ha-ras. The MEK inhibitor PD-98059 was able to block the induction of HB-EGF expression in all the cells, suggesting that MAPK is involved in the pathway of HB-EGF induction by EGF. It is known that ras leads to activation of ERK 1/2 MAPK and that EGF can activate other erbB receptors in the absence of EGFR. We propose that when the cells are stimulated with EGF and the EGFR is blocked, EGF is going to bypass EGFR and stimulate erbB-3/erbB-2 heterodimer formation, which is a secondary event that takes places after EGFR stimulation. This event is not enough to trigger signaling in the nontransformed MCF-10A and in the cells overexpressing the Ha-ras protooncoge (N-ras). However, in the cells transformed with oncogenic Ha-ras, ERK 1/2 MAPK expression is upregulated and induction of HB-EGF can take place after stimulation of EGF through

erbB-2/erbB-3 heterodimers, which are going to trigger MAPK activation and eventually HB-EGF induction of transcription. Future studies are required to prove this hypotheis and to accomplish the remaining of the proposed tasks.

## FIGURE LEGENDS

Fig. 1. Time course of the effects of EGF-related peptides and TPA on HB-EGF mRNA levels. MCF-10A cells were grown as described Experimental Procedures and treated with different growth factors (10 ng/ml) or TPA (100 nM) for 1, 3, 6 or 24 hr. RNA was isolated and analyzed by RNase protection. The bands were quantified by scanning densitometry. The HB-EGF mRNA bands were normalized with the corresponding 36B4 mRNA bands and represented as percentage of control.

Fig. 2. HB-EGF protein levels in MCF-10A cells. MCF-10A cells were induced with EGF (10 ng/ml) for 3, 6, 24, 48 and 72 hr. Subsequently, cells were lysed, conditioned media (CM) were collected and 300 μg of protein from lysates (LYS) or CM were immunoprecipitated with an anti-HB-EGF polyclonal antibody followed by Western blot analysis with other anti-HB-EGF antibody. Sizes in kilodalton are indicated to the left.

Fig. 3. HB-EGF mRNA levels on MCF-10A ras clones. MCF-10A parental cells and the derived ras clones were grown as described in Experimental Procedures. RNA was isolated and analyzed by RNase protection. The HB-EGF and 36B4 bands are indicated.

Fig. 4. Levels of Ha-ras protein in MCF-10A transformed cells. Levels of p21 (Ha-ras) protein were analyed by Western blot in nontransformed MCF-10A cells and in MCF-10A cells transfected with the Ha-ras protooncogene or with a point-mutated Ha-ras form.

Fig. 5. Anchorage-independent growth of MCF-10A transformed cells. Nontransformed and transformed MCF-10A cells were seeded over agar in triplicates and after 14 days, cells were stained and colonies larger than 50 μm were counted. Error bars are the standard error of the mean.

Fig. 6. Levels of EGFR, erbB-2 and erbB-3 in MCF-10A transformed cells: Levels of EGFR, erbB-2 and erbB-3 were measured by Western blot analysis in MCF-10A cells and in MCF-10A transformed with the normal Ha-ras protooncogene (N-ras) and with a point mutated Ha-ras oncogene (T2 series).

Fig. 7. Levels of MAPK and phsopho-MAPK in MCF-10A transformed cells. Levels of p44/42 MAPK (p42) and their phosphorylated forms (pp42/44) were measured by Western blot in MCF-10A cells transfected with Ha-*ras* (N-ras) or an activated form of Ha-*ras* (Ha-ras) from which subclones were derived (T2).

Fig. 8. Levels of MAPF activity in MCF-10A transforemd cells. MAPK activity was measured in nontransformed MCF-10A cells and in MCF-10A cells transfected with the Ha-ras protooncogene (Ha-ras) or oncogenic Ha-ras (Ha-ras) from which subclones were derived (T2). Cells were lysed, 300 μg of protein were immunoprecipitated with an anti-phospho p44/42 MAPK antibody and incubated with Elk-1. Levels of pElk-1 and Elk-1 were determined by Western blot anaysis and bands were quantified by scanning densitometry. The pElk-1/Elk-1 ratio is represented as relative units of MAPK activity.

Fig. 9. Effect of the 225 neutralizing antibody on the EGF induction of HB-EGF mRNA levels. MCF-10A cells and cells transfected with the normal Ha-*ras* protooncogene (N-ras) and subclones derived from cells transfected with an activated Ha-*ras* oncogene (T2) were preincubated with the 225 neutralizing antibody against the EGF receptor (10 μg/ml) and treated with EGF (10 ng/ml) for 1 hr. HB-EGF mRNA levels were analyzed by RNase protection. The bands were quantified by scanning densitometry. The HB-EGF mRNA

bands were normalized with the corresponding 36B4 mRNA bands and represented as percentage of control.

Fig. 10. Effect of DAPH-1 and PD 98059 on the EGF induction of HB-EGF mRNA levels. MCF-10A cells and cells transfected with the normal Ha-*ras* protooncogene (N-ras) and a subclone derived from cells transfected with an activated Ha-*ras* oncogene (T2B) were preincubated with the EGFR inhibitor DAPH-1 (50 μM) or with the MEK inhibitor PD 98059 (30 μM) for 1 hr and treated with EGF (10 ng/ml) for 1 hr. HB-EGF mRNA levels were measured by RNase protection. The bands were quantified by scanning densitometry. The HB-EGF mRNA bands were normalized with the corresponding 36B4 mRNA bands and represented as percentage of control.

Fig. 11. Effect of PD 98059 on phosphorylation of p44/42 MAPK. MCF-10A cells were pretreated with PD-98059 (30 μM) for 30 min prior to treatment with EGF (100 ng/ml) for 10 min. Subsequently, cells were lysed and levels of pp44/42 and p44/42 were determined by Western blot.

Fig. 12. Phosphorylation levels of EGFR in MCF-10A transformed cells. Nontransformed MCF-10A cells and MCF-10A cells transfected with the Ha-ra protooncogene (N-ras) or with a oncogenic Ha-ras (Ha-ras) from which subclones were derived (T2) were stimulated with EGF (100 ng/ml) for 10 min. Cells were lysed, EGFR was immunoprecipitated with an anti-EGFR antibody and subjeceted to Western blot an analysis with anti-P-Tyr antibodies (pEGFR).

Fig. 13. Effect of 225 monoclonal antibody on phosphorylation of EGFR. MCF-10A cells and the T2B ras clone were pretreated with the 225 Ab (10 μg/ml) for 30 min and cells were challenged with EGF (100 ng/ml) for 10 min. Subsequently, cells were lysed and 300 μg of protein were incubated with an anti-EGFR antibody followed by Western blot analysis with anti-P-Tyr antibodies.

Fig. 14. Phosphorylation levels of erbB-2 in MCF-10A transformed cells. Nontransformed MCF-10A cells and MCF-10A cells transfected with the Ha-ra protooncogene (N-ras) or with a oncogenic Ha-ras (Ha-ras) from which subclones were derived (T2) were stimulated with EGF (100 ng/ml) for 10 min. Cells were lysed, erbB-2 was immunoprecipitated with an anti-erbB-2 antibody and subjected to Western with anti-P-Tyr antibodies (perbB-2) or with an anti-erbB-2 antibody (erbB-2).

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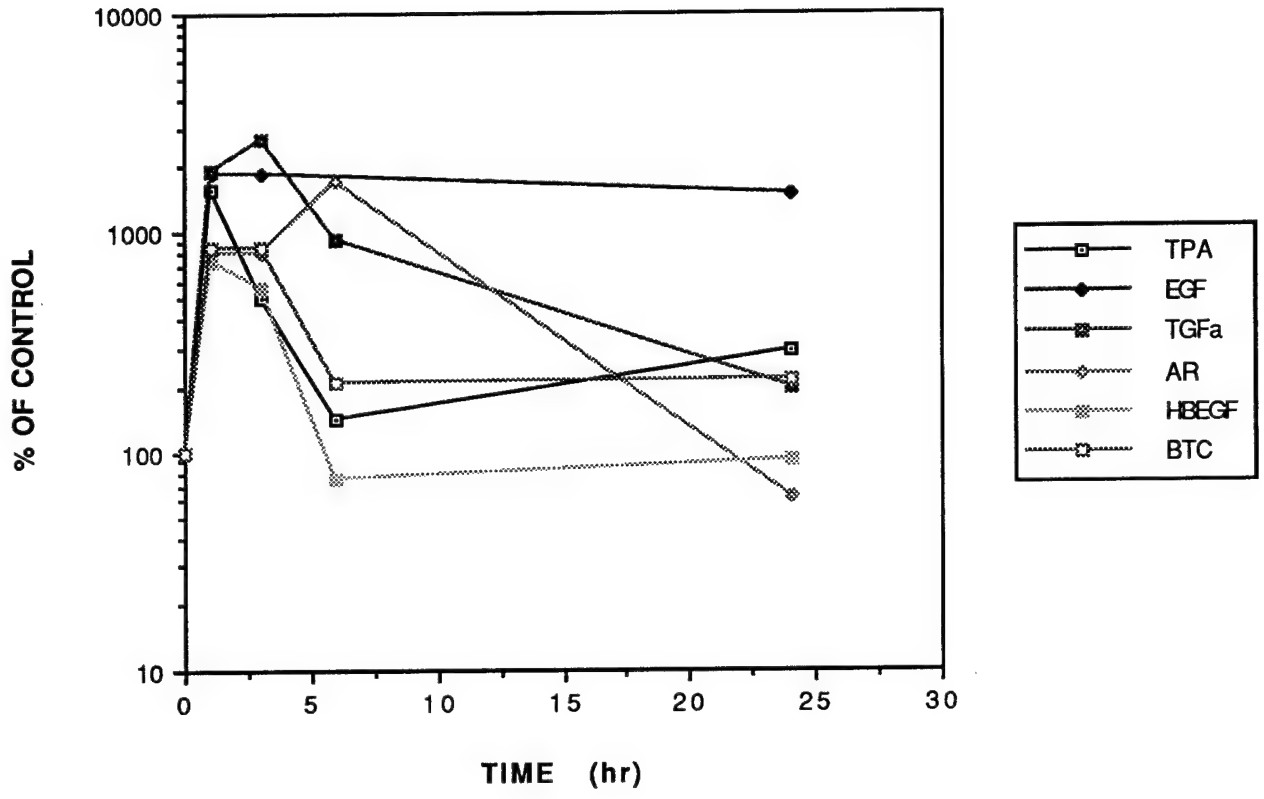
10000
1000
100
10
% OF CONTROL
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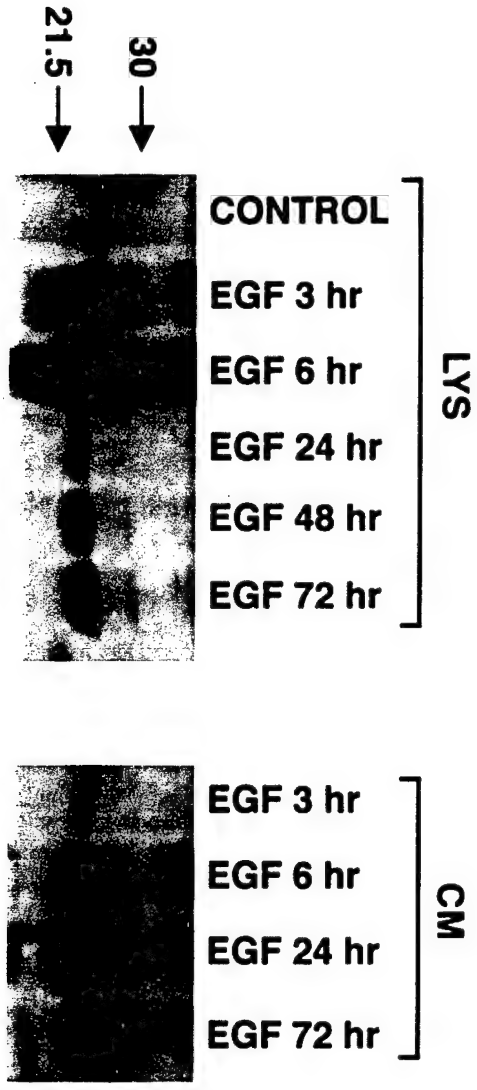
5
10
15
20
25
30
TIME (hr)
TPA
EGF
TGFa
AR
HBEGF
BTC

Figure 1


21.5
30
CONTROL
EGF 3 hr
EGF 6 hr
EGF 24 hr
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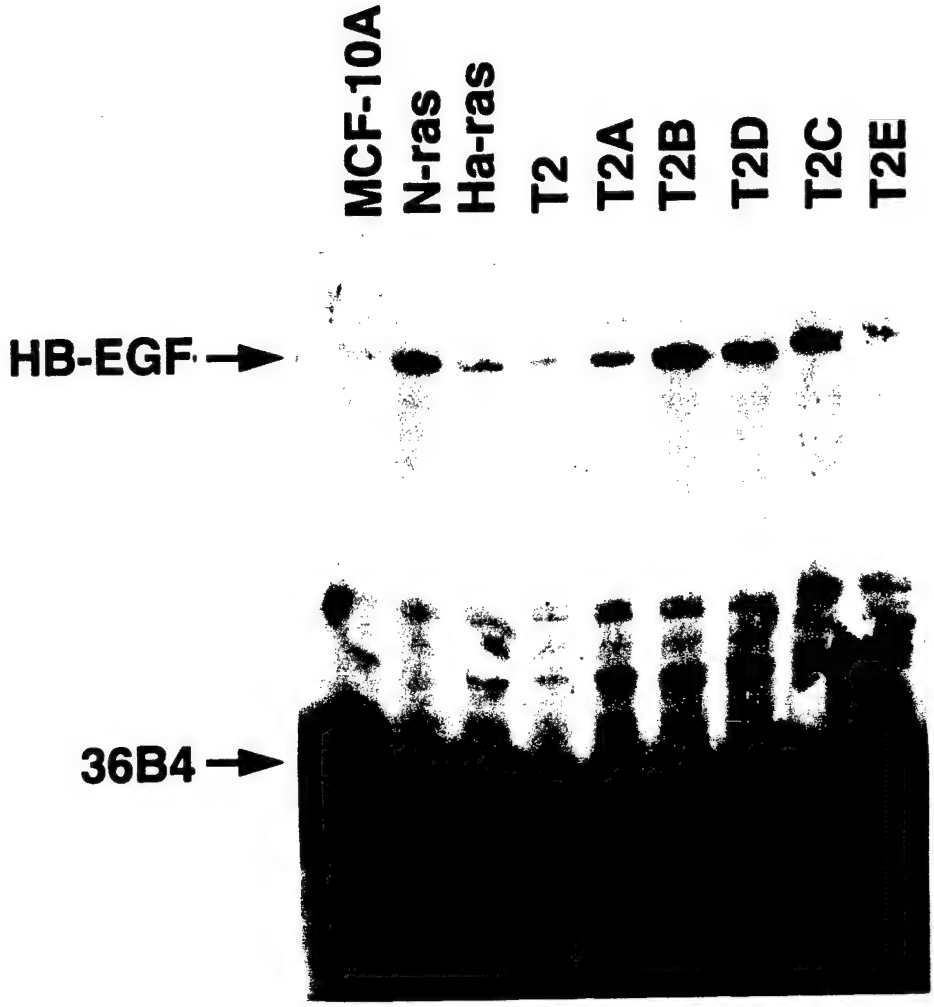
EGF 72 hr
LYS
EGF 3 hr
EGF 6 hr
EGF 24 hr
EGF 72 hr
CM

Figure 2

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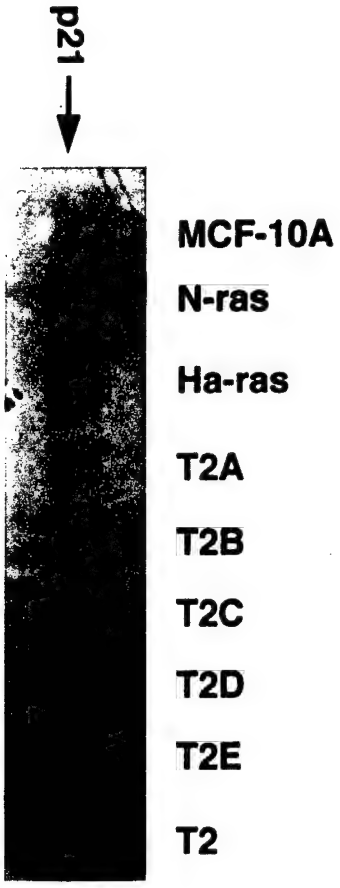


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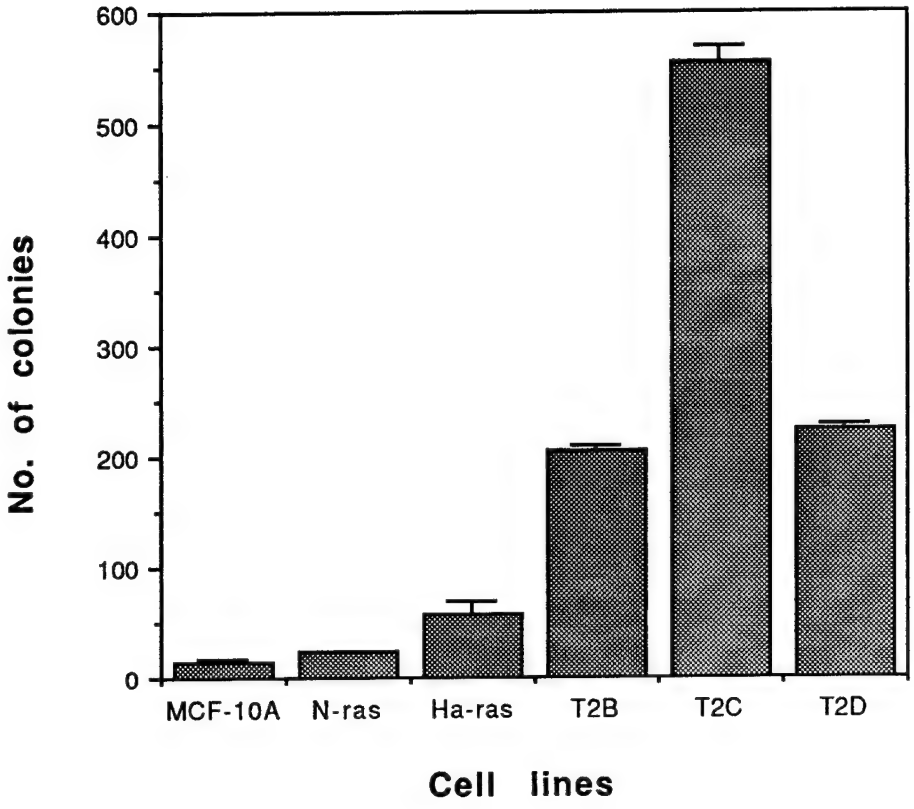
600
500
400
300
200
100
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No. of colonies
MCF-10A
N-ras
Ha-ras
T2B
T2C
T2D
Cell lines

Figure 5

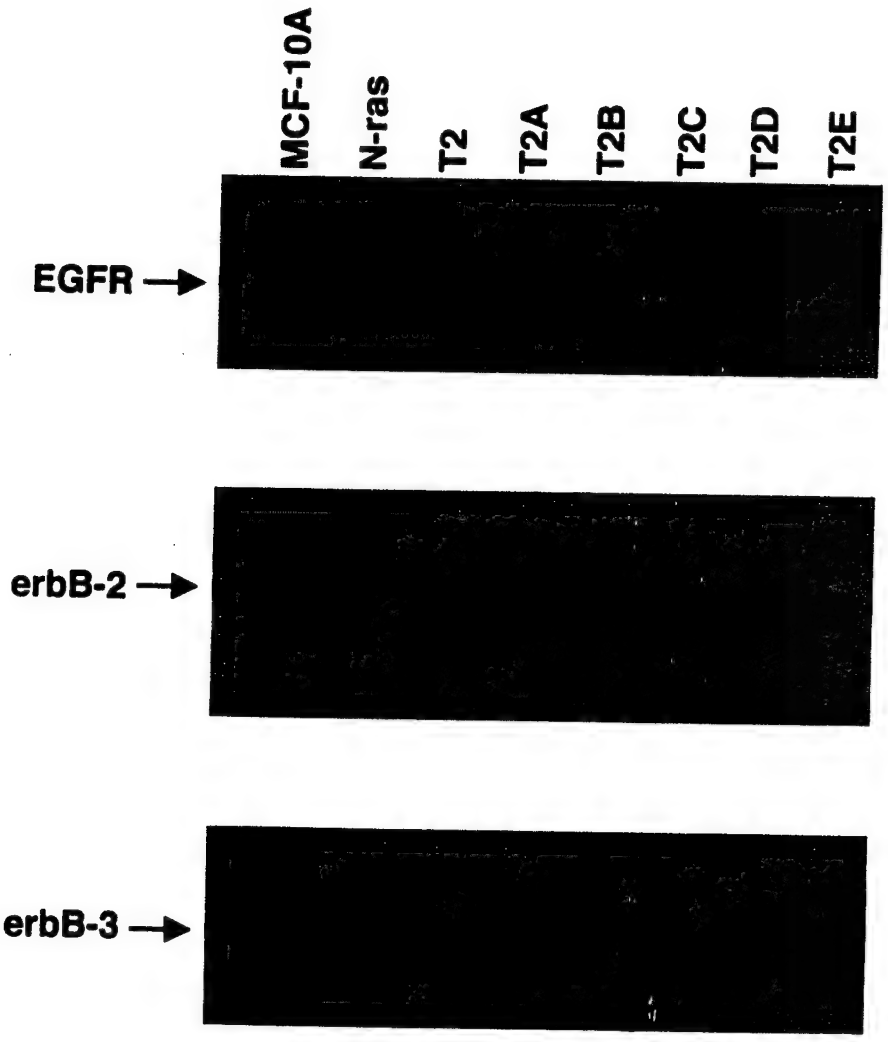
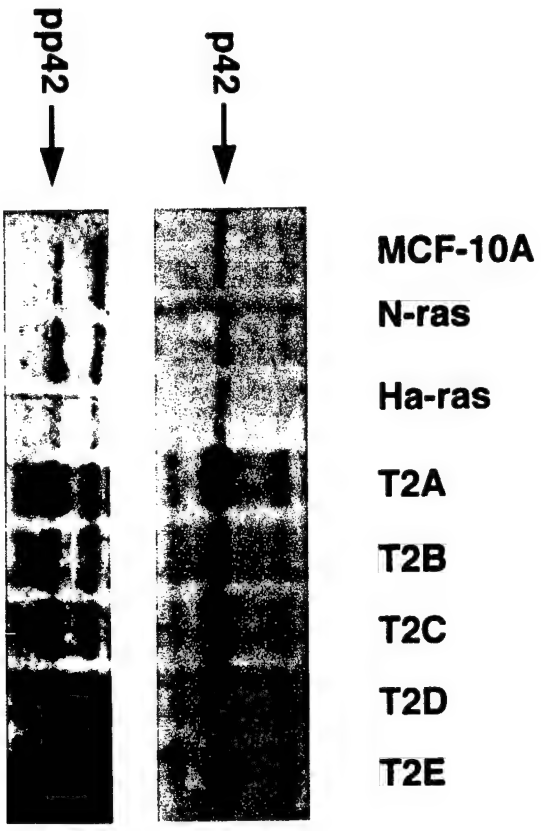



Figure 6

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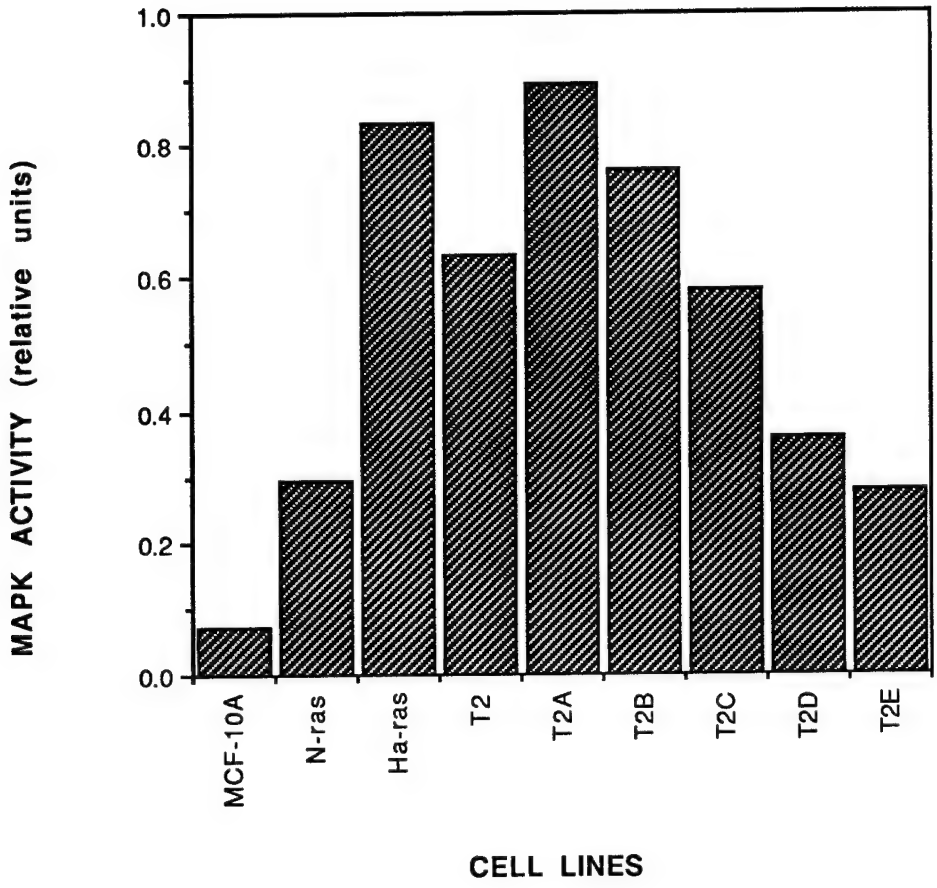
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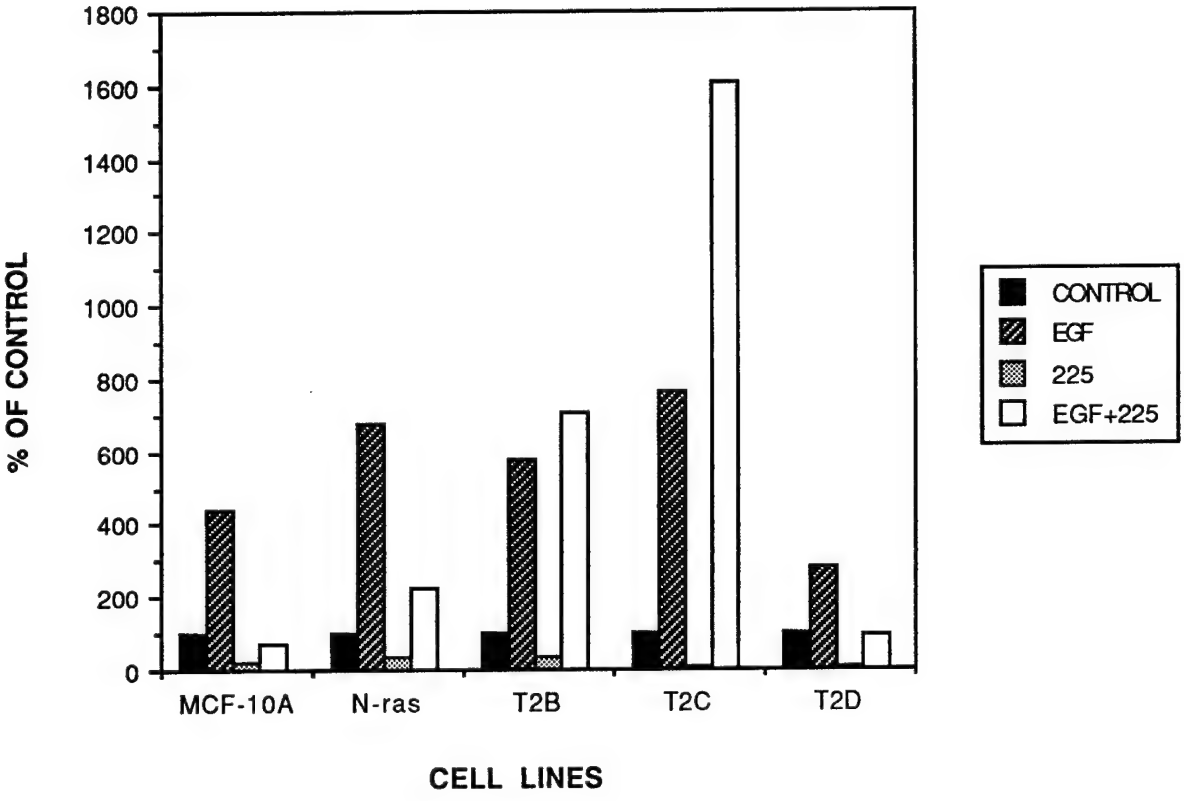
0.6
0.4
0.2
0.0
MCF-10A
N-ras
Ha-ras
T2
T2A
T2B
T2C
T2D
T2E
CELL LINES

Figure 8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1400
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200
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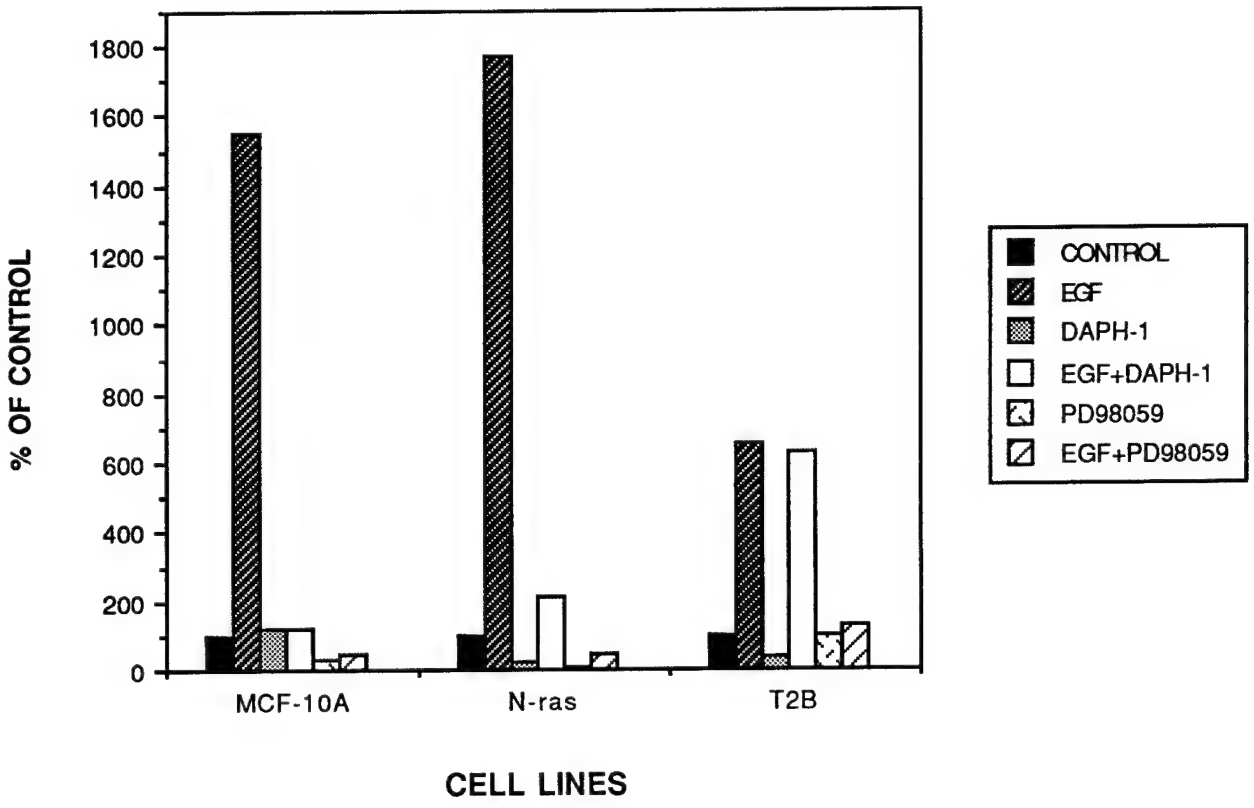
MCF-10A
N-ras
T2B
T2C
T2D
CELL LINES
CONTROL
EGF
225
EGF+225

Figure 9


% OF CONTROL
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1600
1400
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600
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0
MCF-10A
N-ras
T2B
CELL LINES
CONTROL
EGF
DAPH-1
EGF+DAPH-1
PD98059
EGF+PD98059

Figure 10

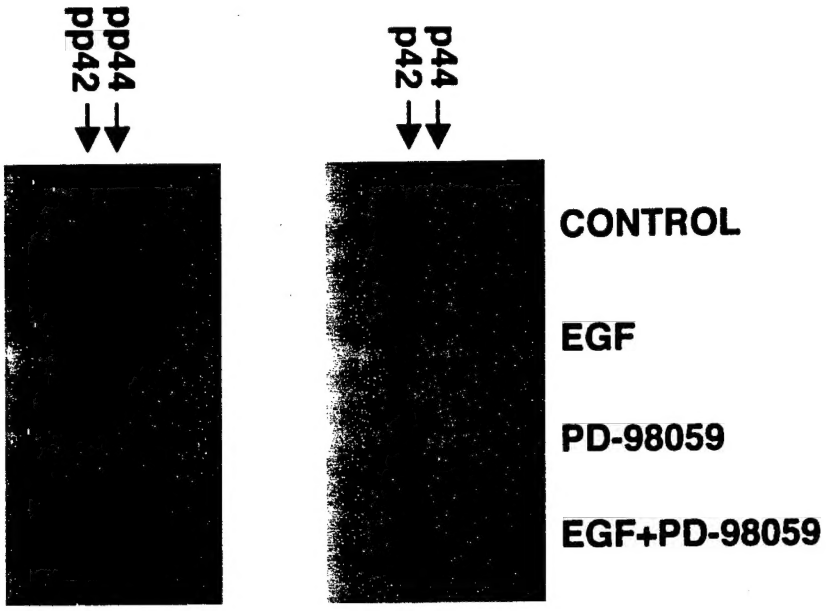


Figure 11

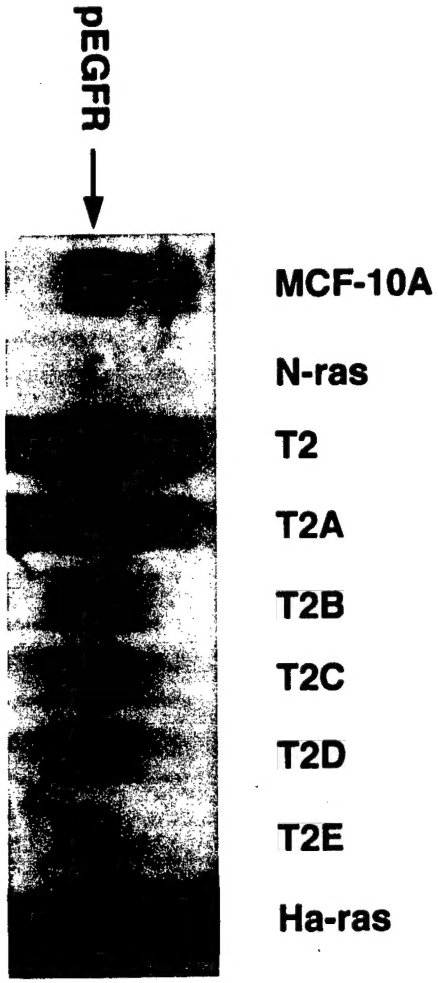


Figure 12

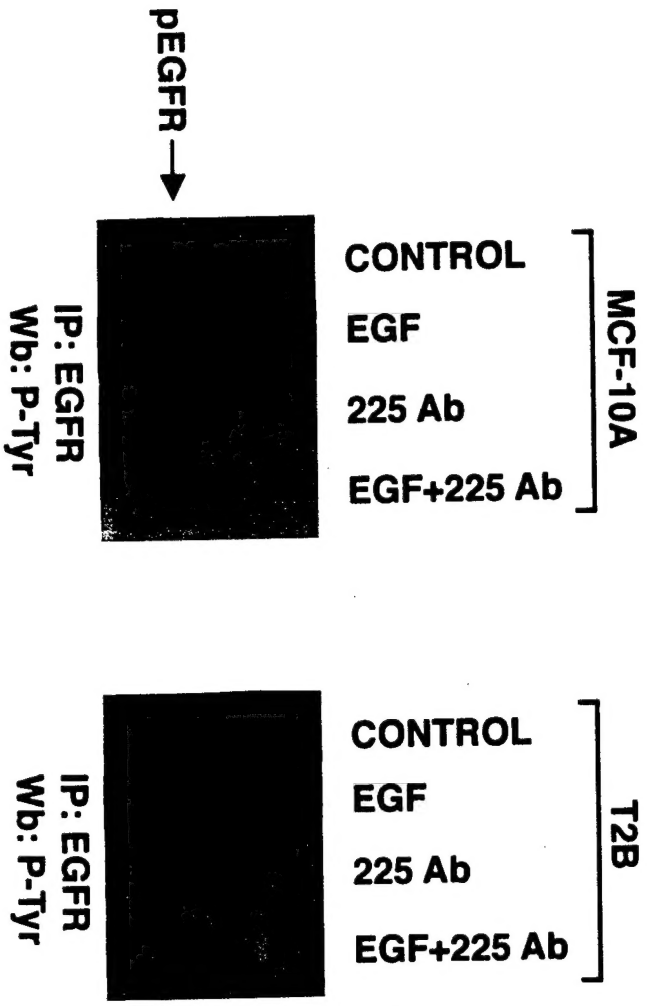
pEGFR
IP: EGFR
Wb: P-Tyr
CONTROL
EGF
225 Ab
EGF+225 Ab
MCF-10A
IP: EGFR
Wb: P-Tyr
CONTROL
EGF
225 Ab
EGF+225 Ab
T2B

Figure 13

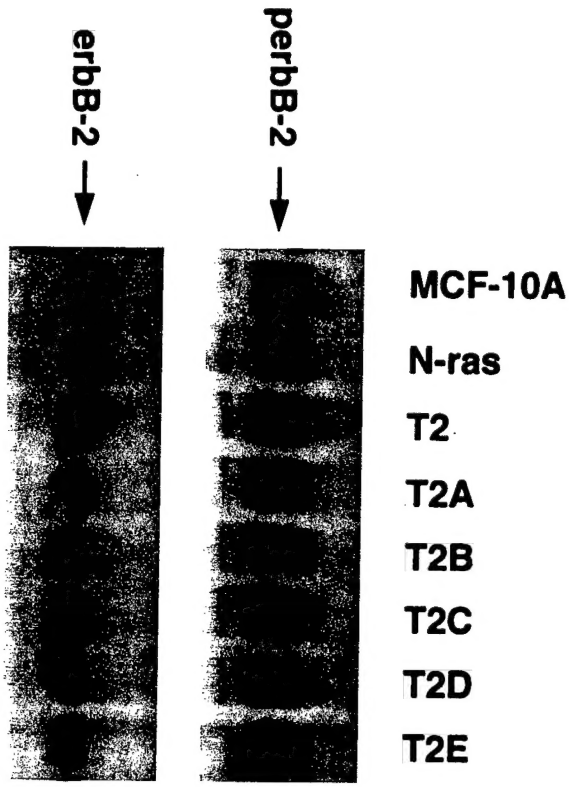


Figure 14